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Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes *in trans*

Carolyn Napoli,[1] Christine Lemieux, and Richard Jorgensen[2]

DNA Plant Technology Corporation, 6701 San Pablo Avenue, Oakland, California 94608

We attempted to overexpress chalcone synthase (CHS) in pigmented petunia petals by introducing a chimeric petunia CHS gene. Unexpectedly, the introduced gene created a block in anthocyanin biosynthesis. Forty-two percent of plants with the introduced CHS gene produced totally white flowers and/or patterned flowers with white or pale nonclonal sectors on a wild-type pigmented background; none of hundreds of transgenic control plants exhibited such phenotypes. Progeny testing of one plant demonstrated that the novel color phenotype co-segregated with the introduced CHS gene; progeny without this gene were phenotypically wild type. The somatic and germinal stability of the novel color patterns was variable. RNase protection analysis of petal RNAs isolated from white flowers showed that, although the developmental timing of mRNA expression of the endogenous CHS gene was not altered, the level of the mRNA produced by this gene was reduced 50-fold from wild-type levels. Somatic reversion of plants with white flowers to phenotypically parental violet flowers was associated with a coordinate rise in the steady-state levels of the mRNAs produced by both the endogenous and the introduced CHS genes. Thus, in the altered white flowers, the expression of both genes was coordinately suppressed, indicating that expression of the introduced CHS gene was not alone sufficient for suppression of endogenous CHS transcript levels. The mechanism responsible for the reversible co-suppression of homologous genes *in trans* is unclear, but the erratic and reversible nature of this phenomenon suggests the possible involvement of methylation.

INTRODUCTION

Anthocyanins are flavonoid compounds derived from the phenylpropanoid pathway that are largely responsible, among other functions, for plant coloration, especially in flowers. Chalcone synthase (CHS) is the key enzyme in flavonoid biosynthesis. It catalyzes the condensation of one molecule of 4-coumaroyl-CoA with three molecules of malonyl-CoA to form naringenin chalcone, which is the central intermediate in the biosynthesis of flavonols, flavones, isoflavonoids, and anthocyanins. CHS cDNA and genomic clones have been isolated from a number of plant species (summarized in Niesbach-Klosgen et al., 1987; Koes et al., 1989b). In petunia, CHS comprises a multigene family (Koes et al., 1987) in which only one gene is expressed to high levels in petal tissue (Koes et al., 1989a). In maize, CHS has been shown to be rate limiting for anthocyanin production (Coe and Neuffer, 1977; Dooner, 1983), although it is not limiting in *Antirrhinum* (Sommer et al., 1988). We have introduced a chimeric CHS gene into petunia in an attempt to overexpress CHS and to test whether this enzyme is rate limiting to anthocyanin biosynthesis. The results of these experiments show that introduction of the chimeric gene can block anthocyanin biosynthesis by co-inhibiting endogenous and introduced CHS gene expression.

[1] Current address: Department of Environmental Horticulture, University of California, Davis, CA 95616.

[2] To whom correspondence should be addressed.

RESULTS

Introduction of a CHS Transgene Inhibits Anthocyanin Pigmentation

A chimeric CHS gene was constructed in which a cauliflower mosaic virus 35S promoter was fused to the coding sequence of a CHS cDNA clone and was introduced via *Agrobacterium* into three petunia genotypes: a commercial hybrid variety (Pink Cascade), a pale pink inbred (R18), and a deep violet inbred (V26) (see Methods). Of six Pink Cascade transgenotes (i.e., transgenic plants), three produced pure white flowers, two produced patterned flowers characterized by small pink nonclonal wedges at the petal

tips on otherwise pure white flowers, and one produced pale variegated flowers with many small nonclonal sectors. Five of 14 transgenotes of R18 produced flowers with nonclonal white sectors of various sizes. Nine of 28 transgenotes of V26 produced either pure white flowers or a variety of novel patterns. No transgenotes in any of the genotypes produced flowers that were visibly darker than the parental genotype. We have never observed altered flower color phenotypes in our experience with hundreds of petunia transgenotes derived from these varieties and not carrying introduced CHS transgenes.

Experiments previously reported by van der Krol et al. (1988) using a similar, but independently derived, chimeric CHS gene did not result in any phenotypic change in flower color. To determine whether the chimeric CHS gene we had introduced might have been altered during the introduction of restriction sites by oligonucleotide mutagenesis, we sequenced the coding region of the construct (C. Napoli, J. Villanueva, and P. Muhlrad, unpublished results) and compared it with the published sequence of the cDNA clone from which it was derived (Niesbach-Klosgen et al., 1987). No differences were found. In addition, the length of the polypeptide encoded by the construct was determined by fusion to bacterial expression signals and performing protein gel blot analysis on bacterial extracts (C. Napoli, K. Greisen, and J. Villanueva, unpublished results). The length of the polypeptide was the same as in petunia. We conferred with van der Krol and co-workers to identify the differences in our experiments that might explain the different results. As described in the accompanying paper by van der Krol et al. (1990b), they subsequently found that white sectors could be observed in their plants by placing the plants under supplementary light (see also van der Krol et al., 1990a). This association between color phenotype and supplementary light in Amsterdam could be due to differences in the environment between Oakland and Amsterdam, differences in host genotypes, and differences in transgene constructs.

Variation in Phenotypes of CHS Transgenotes

The normal phenotype of the deep violet flowers of inbred V26 and examples of the flower phenotypes caused by the CHS sense transgene in four V26 transgenotes are shown in Figure 1. Two classes of patterns were observed: a "wedge" pattern (plant 218.11) and a radial, star-like ("Cossack dancer") pattern (plant 218.43). The "dancer" class of patterns includes irregular patterns, as can be seen on plants 218.38 and 218.56. The white sectors of the wedge and dancer patterns are not clonal sectors.

The flower patterns elicited by the CHS transgene often vary among the flowers of a single plant. The extent of the variation observed among flowers on individual transgenic plants is also illustrated in Figure 1. On some transgenotes, such as 218.11 and 218.43 (the "type" plants of the wedge and dancer classes, respectively), variation was limited to increases and decreases in the size of white sectors. White sectors were typically larger in summer and smaller in winter, similar to the well-known "star" and "picotee" patterns of certain commercial petunias. Similarities between the transgene-induced phenotypes reported here and these naturally occurring petunia phenotypes are discussed in more detail in the accompanying paper by van der Krol et al. (1990b). The sharpness of the boundaries between white and pigmented sectors in all wedge patterns and in the most regular dancer patterns (218.43) does not vary significantly and is several cells wide. However, in irregular dancer patterns (e.g., plants 218.38 and 218.56), the sharpness of these boundaries varies a great deal and is usually very diffuse. None of the wedge-class plants (three in the V26 genotype, five in R18, and two in Pink Cascade) has ever shown very diffuse boundaries.

The patterns we have observed were somewhat stable in the sense that flowers on the same branch were typically affected to the same degree, i.e., white sectors were similar in size and pattern. Occasionally, flowers on different branches were affected to a different degree. Changes in the degree of pigmentation during plant growth were frequently discontinuous and often occurred on side branches immediately after branching. The most dramatic discontinuous changes in pigmentation occurred in the two solid white transgenotes 218.38 (Figure 1) and 218.41 (not shown in Figure 1). Both of these plants produced hundreds of pure white flowers for 4 months before producing a branch with pigmented flowers. These pigmented flowers on the new branch of plant 218.38 were in the dancer class. The flowers on the new branch of plant 218.41 were entirely violet with the same deep intensity of V26, i.e., they were phenotypically revertant (except that on some revertant flowers a small, white spot was produced). Plants grown from cuttings taken from violet-flowered and white-flowered branches of 218.41 have produced hundreds of flowers of their respective colors over a period of 6 months without further alteration in color. These vegetative propagules of 218.41 provide a valuable resource for investigating the CHS transgene effect at the molecular level because they eliminate the effect of patterns and the need to dissect flowers.

Heritability of Phenotypes

First-generation out-cross progeny of one of the Pink Cascade transgenotes crossed to V26 were scored for kanamycin resistance and flower color. Kanamycin resistance co-segregated with the novel, patterned flower phenotype (five plants), and all 13 kanamycin-sensitive plants were found to exhibit normal, unaltered flower color. Thus, the introduced T-DNA was correlated with the novel color phenotype.

Three of the V26 primary transgenotes pictured in Figure

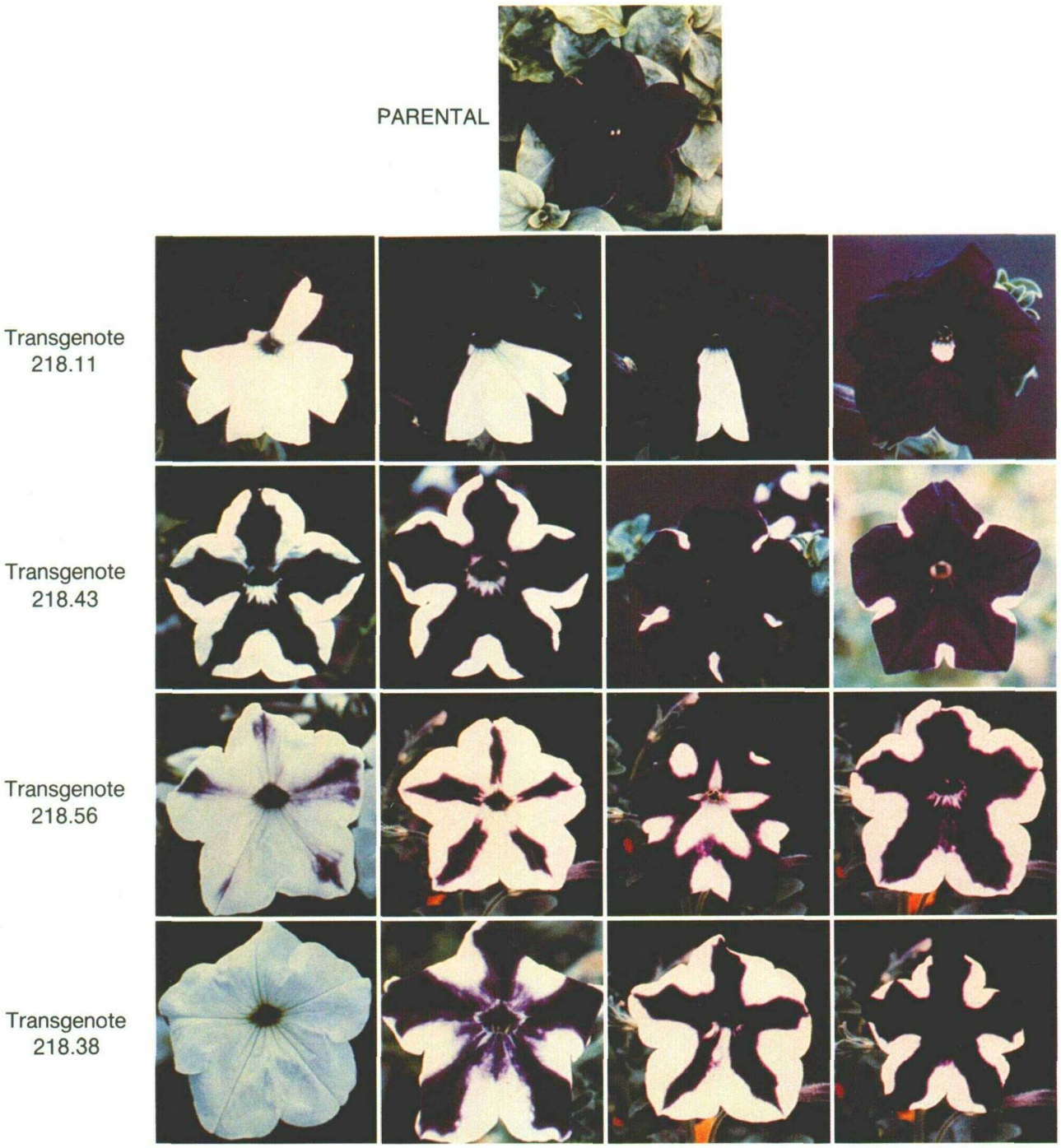

Figure 1. Phenotypes of Chimeric CHS Transgenotes and Variations among Flowers on Single Plants.

A control (parental) V26 flower is shown along with four different CHS transgenotes. Four representative flowers are shown in a row for each of four transgenotes, identified at the left of each row.

Table 1. Segregation of Phenotypes in Out-Cross Progeny

	Male Parent					
	V26		M1		R27	
Transgenote	P[a]	W[b]	P	W	P	W
218.18	37	0	44	9	46	3
218.43	24	16	17	12	19	19
218.56	24	18	49	0	25	21
218.38	21	31	27	27	27	28

[a] Flowers fully pigmented.
[b] Flowers white or with white sectors.

1 and one other V26 primary transgenote (218.18) exhibiting wedge patterns similar to those of 218.11 were back-crossed to V26 and, in parallel, out-crossed to pigmented inbred lines M1 and R27 to determine the heritability of the patterned phenotypes. Table 1 shows the segregation of flower color phenotypes in these populations. All populations displayed segregation ratios consistent with a dominant, single locus transgene with either complete, partial, or no penetrance (although aberrant transmission is also a possibility). For example, segregation of the CHS transgene phenotype of 218.38 suggests essentially complete penetrance of the phenotype in all crosses, whereas segregation of the transgene phenotype of 218.18 suggests very low penetrance in all crosses and that of 218.56 suggests substantially complete penetrance in two populations and no penetrance in the third population. The reason for this apparent variation in penetrance among populations of progeny remains to be determined. Important factors might include the location of the transgene in the genome and the genotype of the out-cross parent.

All progeny of 218.38 expressing a novel color phenotype were pure white just like their parent. Eight of these progeny were observed over a period of time and all eventually produced branches with irregular dancer patterns, but only after several months of growth, as in their parent (218.38). Progeny of 218.43 produced a wide range of flower phenotypes, as shown in Figure 2. Most of these were obviously related to the parental dancer phenotype, but none was exactly like this parental phenotype with its sharp, regular boundaries. In fact, in all progeny of 218.43, this pattern has decayed into less regular patterns, including very disorganized, "tie-dyed" patterns. Progeny of 218.56 also produced a wide range of flower phenotypes similar to the progeny of 218.43 (Figure 2). More recently, we have observed the wedge pattern in one of the progeny of 218.56.

In progeny plants, as in primary transgenotes, flowers on the same branch had similar patterns and sizes of sectors, and different branches often produced flowers with different pattern regularity or different-sized sectors. Both primary transformants and progeny plants with irregular patterns have been observed to produce flowers with more regular patterns and vice versa. To summarize our observations on the variability of patterns, we can state

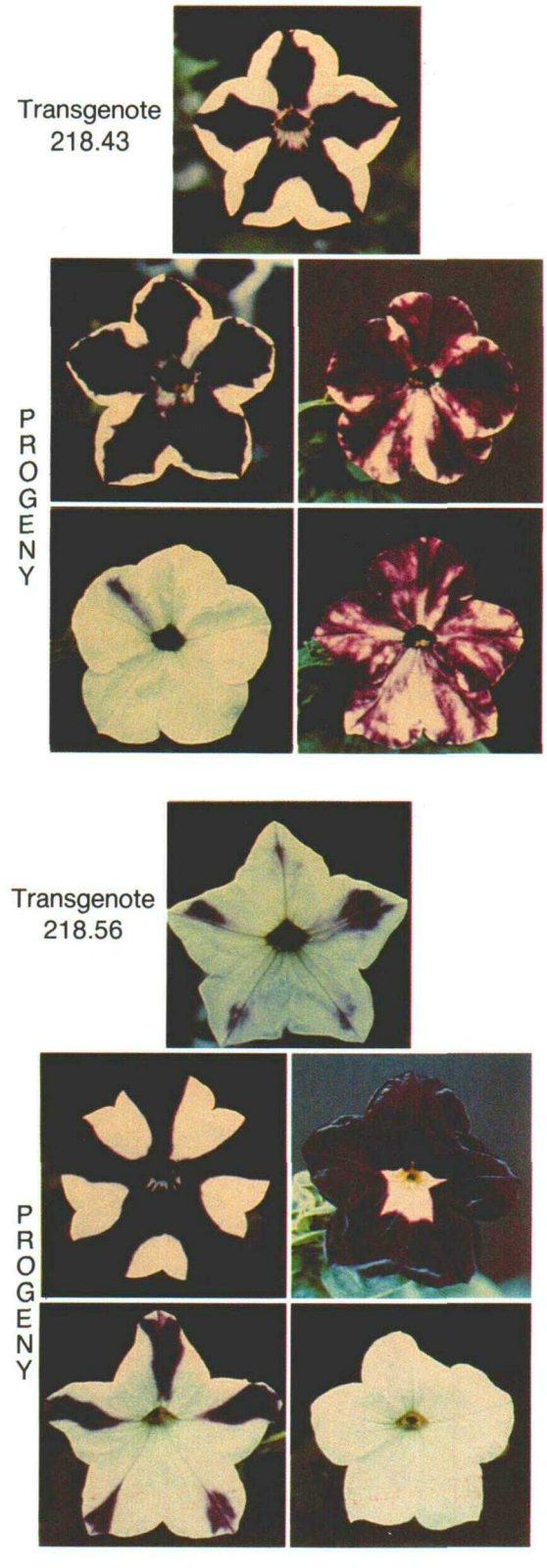

Figure 2. Heritability and Variation among Progeny of CHS Transgenotes Back-Crossed to V26 (Parental).

A flower from each of four different progeny plants from back-crosses to V26 of transgenotes 218.43 and 218.56 are shown.

that substantial variability was observed both within and among progeny plants with respect to sector size, pattern regularity, and sharpness of the boundaries between colored and white sectors.

Message Levels of CHS Transgene and Endogenous Gene

The steady-state mRNA levels produced by both endogenous and introduced CHS genes were analyzed in petunia flowers by RNase protection experiments. The number of CHS genes in V26 is unknown; however, Koes et al. (1989a) have shown eight genes in variety V30, one of which accounts for 90% of CHS expression. Petals were harvested at multiple stages of development from normal violet-colored flowers of a transgenic V26 control plant that does not carry a CHS transgene and pure white flowers of transgenote 218.38. Figure 3 shows an autoradiogram of an RNase protection experiment performed on control petal RNAs that contain only the endogenous CHS message(s). In the control flowers, the steady-state CHS message level gradually increased in abundance up to the 40-mm flower bud and then declined as the flower matured.

Figure 4 shows an autoradiogram of 218.38 petal RNAs (lanes 4 to 9) that express both the transgene and the endogenous gene(s). To compare this experiment with the control experiment, RNA from a 40-mm control flower bud was run in lane 3 of Figure 4. In flowers of 218.38, the message level of the predominantly expressed endogenous CHS gene followed essentially the same pattern of

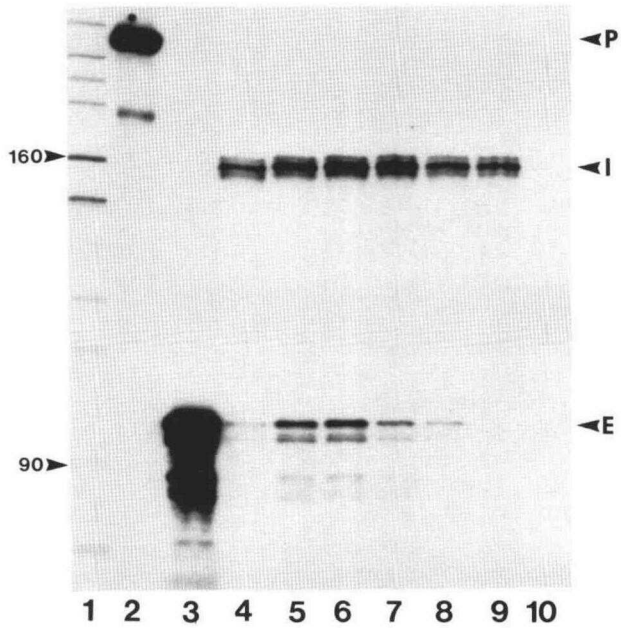

Figure 4. Developmental Pattern of Expression of Introduced and Endogenous CHS Messages in White Flowers of Transgenote 218.38.

Lane 1 is the same molecular weight marker as in Figure 3. Lane 2 is the undigested probe. Lane 3 is a 40-mm corolla control RNA similar to lane 4 in Figure 3. Lanes 4 through 9 contain RNase-protected RNA from corollas of 218.38 flowers 15 mm, 30 mm, 40 mm, 53 mm, and 58 mm (two samples) in length, respectively. Lane 10 is a tRNA negative control. The arrows indicate the positions of the endogenous (E) CHS-protected fragment at 96 bases, the introduced (I) CHS-protected RNA at 157 bases, the radiolabeled probe (P) at 208 bases, and two marker fragments at 160 and 90 bases.

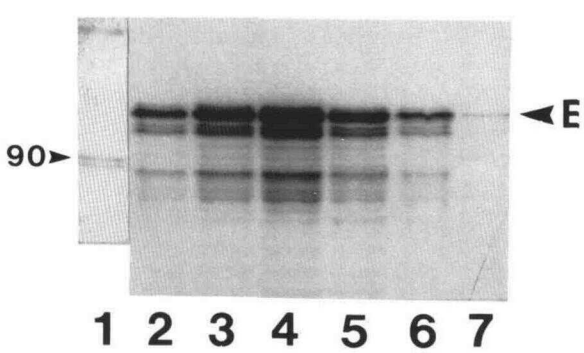

Figure 3. Developmental Pattern of Expression of the Endogenous CHS Message(s) in Violet Flowers from Control Plants.

Lane 1 is a molecular weight marker that is pBR322 DNA digested with HpaII and end labeled. The 90-base fragment is indicated. Lanes 2 through 7 contain RNase-protected RNA from corollas of control flowers of 15 mm, 30 mm, 40 mm, 53 mm, 58 mm, and 58 mm in length, respectively. E indicates the position of the endogenous CHS-protected fragment at 96 bases.

expression during development as control flowers; however, the overall message level at any stage in development was reduced approximately 50-fold relative to the level in the comparable stage in the control plant. The message produced by the introduced CHS transgene was present at a nearly constant level in all stages of petal development assayed, several times higher than peak expression levels from the endogenous CHS gene in 218.38 but far below peak expression levels of the endogenous CHS gene in the control plant.

We have also performed RNase protection analyses with a chalcone isomerase probe to determine whether the expression of the gene controlling the enzymatic step immediately following CHS was affected by the CHS transgene. No difference was observed between the levels of chalcone isomerase message in control violet flowers and transgenote 218.38 white flowers (P. Muhlrad and C. Napoli, unpublished results).

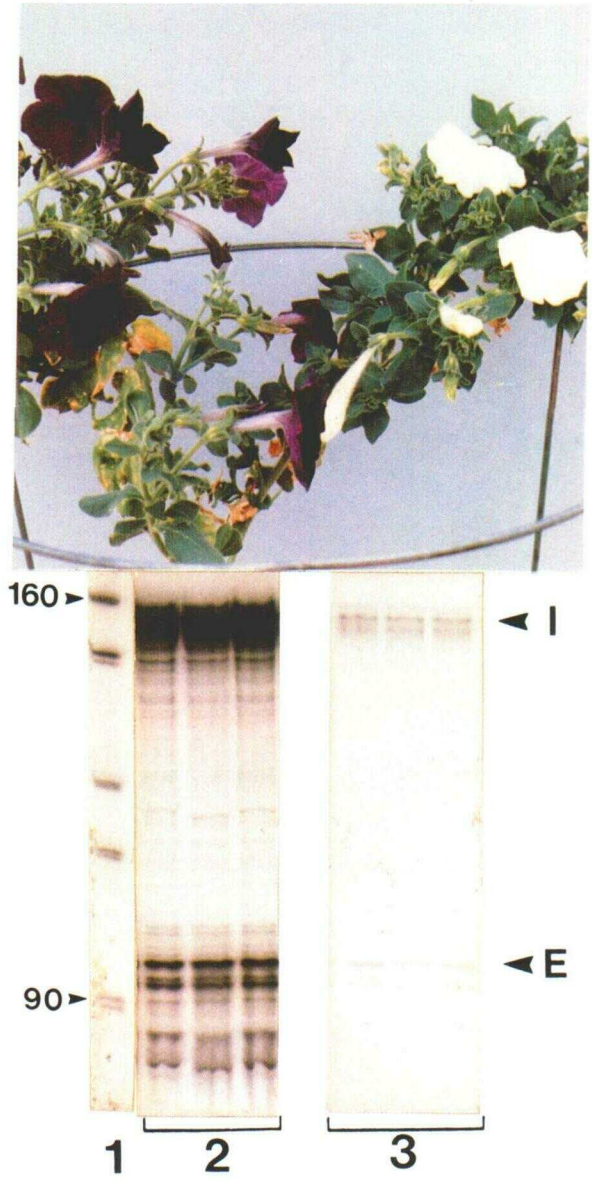

Figure 5. Comparison of Steady-State CHS Message Levels in Violet and White Flowers from Transgenote 218.41.

RNase protections for violet and white flowers are shown under the corresponding branches of transgenote 218.41. Lane 1 is the end-labeled pBR322 molecular weight standard; the arrows indicate 160 and 90 bases. Lanes labeled "2" are RNase protections of RNA isolated from three separate 40-mm-long violet revertant corollas. Lanes labeled "3" are RNase protections of RNA isolated from three separate 40-mm-long white corollas. E indicates the position of the protected fragment for the endogenous CHS transcript and I indicates the position of the protected fragment for the introduced CHS transcript.

CHS Message Levels in Revertant Flowers

As noted above, plants 218.38 and 218.41 produced solid white flowers for many months before growing branches with patterned (218.38; Figure 1) or solid violet (218.41; Figure 5) flowers. Plants grown from cuttings taken from violet-flowered and white-flowered branches of 218.41 have stably maintained their colors for many months. Steady-state message levels of both the endogenous and introduced CHS genes were examined in these solid white and solid violet flowers. The results presented in Figure 5 demonstrate that both the introduced and endogenous CHS genes produced messages at 30-fold to 50-fold higher levels in the revertant violet flowers of plant 218.41 than in white flowers of plant 218.41. Thus, in white flowers *the message levels of the transgene and the endogenous* CHS gene were coordinately and reversibly suppressed.

Figure 6 shows the developmental pattern of expression for the CHS genes in revertant violet flowers from transgenote 218.41. Lane 1 is from a 40-mm control flower showing the peak level of expression in normal flowers. For the endogenous gene, both the developmental pattern and the peak level of expression were essentially the same in 218.41 revertant flowers as in the control flowers shown

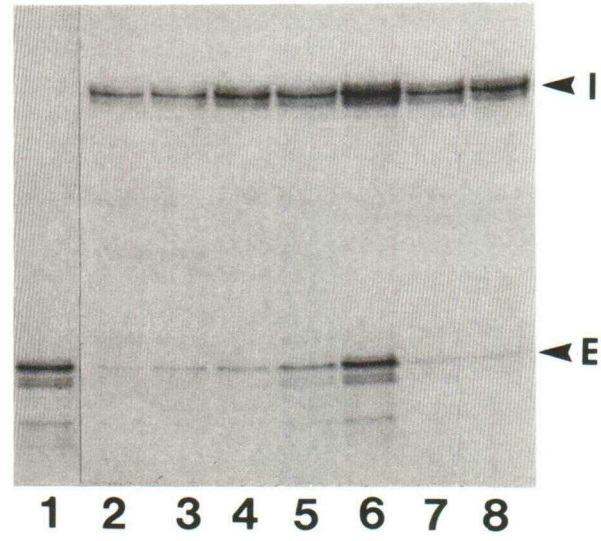

Figure 6. Developmental Expression of the Endogenous CHS Gene in Revertant Violet Flowers from Transgenote 218.41.

Lane 1 is an RNase protection performed on RNA isolated from a 40-mm-corolla from the control plant. Lanes 2 through 8 are RNase protections performed on RNA isolated from violet revertant corollas from transgenote 218.41 at 6 mm, 11 mm, 15 mm, 20 mm, 40 mm, and 70 mm (two samples), respectively. E indicates the position of the protected fragment for the endogenous CHS transcript and I indicates the position of the protected fragment for the introduced CHS transcript.

in Figure 3. Interestingly, the expression of the transgene also appeared to be developmentally regulated, following the same pattern as the endogenous gene except that the message levels at early and late times in development were not as low relative to the peak level as for the endogenous gene. This suggests that, in revertant violet flowers, the CHS transgene is being induced coordinately with the endogenous gene in addition to being constitutively expressed via its 35S promoter. Measurements of the expression of other 35S gene fusions in petals must be undertaken to determine whether or not this is an effect specific to the CHS transgene.

Flowers of transgenote 218.43 (dancer) representing the extremes for white sector size (similar to the extreme flowers of 218.43 shown in Figure 1) were also analyzed by RNase protection to determine CHS message levels. The results indicate that, in patterned flowers, the message level of both the endogenous gene and the transgene in RNA samples isolated from whole flower buds was the same as if RNAs from violet and white sectors were simply added together in proportion to the relative size of sectors (C. Napoli, data not shown). This suggests that message levels in white or violet sectors are the same as in white or violet flowers of 218.41. Similar experiments with progeny plants confirmed this result (C. Napoli, data not shown). Thus, message levels of both the endogenous and the introduced genes vary coordinately proportionate to the degree of pigmentation in patterned flowers. These data support the previous conclusion that the introduced and endogenous genes are expressed in coordinate, reversible fashion.

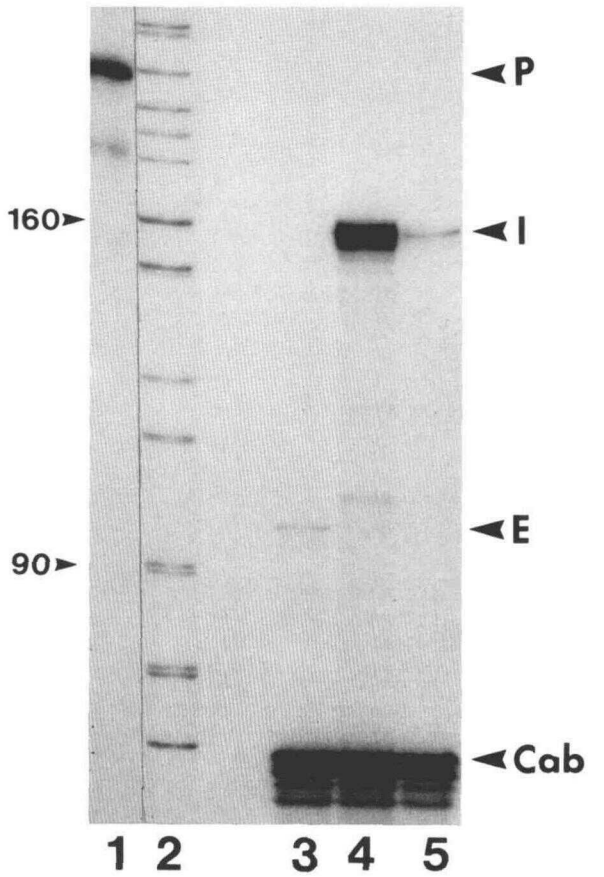

Figure 7. Expression of CHS genes in Leaves of Revertant Violet and White Branches of 218.41.

Lane 1 is the radiolabeled probe, lane 2 is the end-labeled pBR322 molecular weight probe with 90 and 160 bases indicated, lane 3 is RNA isolated from leaf tissue from an untransformed V26 wild-type plant, lane 4 is RNA isolated from leaf tissue from the revertant violet branch, and lane 5 is RNA isolated from the white branch. P indicates the position of the undigested probe, I indicates the protected fragment corresponding to the introduced CHS transcript, E indicates the protected fragment for the endogenous CHS transcript, and Cab indicates the protected fragment for the chlorophyll *a*/*b*-binding protein transcript.

CHS Message Levels in Leaf Tissue

RNase protection analysis was done on young leaf tissue from untransformed wild-type V26 and violet-flowered and white-flowered branches of 218.41. The results are presented in Figure 7. Leaves of both wild-type V26 and violet-flowered 218.41 expressed a low level of endogenous CHS message, whereas there was no detectable message observed in the white-flowered 218.41 leaf tissue. The introduced gene was strongly expressed in 218.41 leaf tissue from the violet revertant branch, whereas, in leaf tissue from the white-flowered branch of 218.41, its expression was reduced by the same proportion as in white petals. Thus, the co-suppression effect also occurs in leaves, which express CHS at much lower levels than do petals.

Patterns Induced by CHS Antisense Transgene

Transgenic V26 plants transformed with an antisense CHS construction are shown in Figure 8. The phenotypes with an antisense CHS gene were not the same as the sense phenotypes either in the degree of pigment reduction or in the nature of the patterns. Whereas the antisense transgene affected only the limbs of the flower corolla, the sense transgene affected all pigmented floral parts (corolla limbs, corolla tubes, and anthers), as well as stems and leaves. The dancer- and wedge-class patterns, which are characteristic of CHS sense transgenotes, have never been seen in antisense flowers. Similarly, the patterns generated with an antisense CHS gene that were presented by van der Krol et al. (1988) differed from the patterns generated with a sense CHS gene in the accompanying paper by van der Krol et al. (1990b).

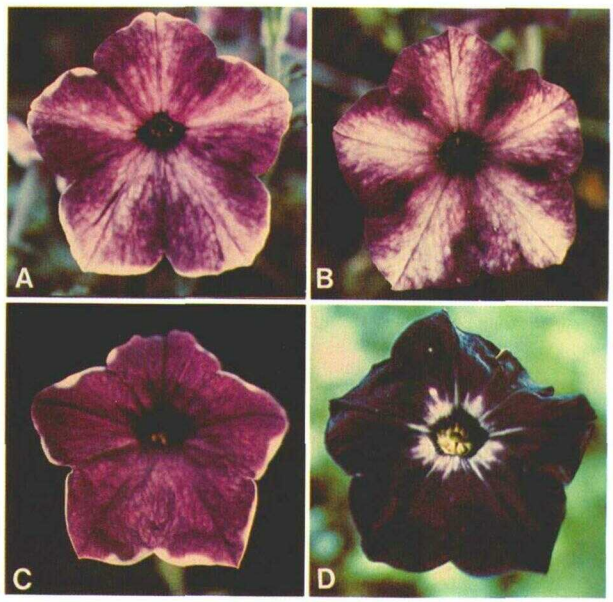

Figure 8. Phenotypes of CHS Antisense Transgenotes.

Four representative flower types resulting from the introduction of the CHS gene in an antisense orientation are shown.
(A) to **(D)** Flowers from independent transgenotes.

DISCUSSION

We have shown, unexpectedly, that the introduction of a CHS transgene resulted in a dramatic reduction in the expression of a homologous gene in a large proportion of independent transgenotes in three different genetic stocks. The fact that a large proportion of independent transgenotes were affected in this manner implies that this phenomenon is an interaction between homologous sequences that can occur *in trans* over great genetic distances. The observation that the degree of this effect varied considerably among transgenotes suggests that the relative positions of the transgene and the endogenous gene in the genome control this variation.

The mechanism for this *trans* interaction is, however, unclear. The most informative result available at this point is that, in phenotypically revertant flowers, transgene expression rose concomitantly with endogenous gene expression. In other words, the effect was both reversible and coordinate. This information allows us to conclude that expression of the transgene is not by itself sufficient to elicit the effect, although it does not address the question of whether expression of the transgene is necessary for suppression of endogenous gene activity. The fact that transcription was not sufficient suggests direct or indirect interactions between homologous genes, probably across considerable genomic distances. Because of the coordinate nature of this interaction and its effect on expression, we have coined the term "co-suppression" to refer to the phenomenon.

Importantly, van der Krol et al. (1990b) have shown that introduction of an intact genomic CHS gene and another flavonoid gene, dihydroflavonol 4-reductase (DFR), can also specifically inhibit endogenous CHS and DFR gene activities, respectively. Furthermore, these workers have observed coordinate, reversible suppression of the DFR transgene and the endogenous DFR gene. Thus, co-suppression is not restricted to chimeric genes or to the CHS gene.

Novel Patterns Associated with Co-Suppression

A phenomenon that must be explained before the regulation of CHS gene expression can be understood fully is the formation of color patterns in flowers carrying the CHS transgene. In particular, it is difficult at present to understand why two particular classes of patterns were observed (dancer and wedge) and why the dancer class appeared in two extremes: a highly regular, "Cossack dancer" pattern and a seemingly chaotic "tie-dyed" pattern (see progeny of 218.43 in Figure 2). The apparent capability of a plant expressing a stable, regular pattern, such as plant 218.43, to exhibit chaotic behavior in progeny plants would require only that some aspect of CHS gene expression must exhibit sensitive dependence on initial conditions (Glass and Mackey, 1988).

Also needing explanation is the observation that the patterns produced by antisense CHS transgenes were different from those produced by sense CHS transgenes. This result would suggest that transcription is involved in at least modifying the co-suppression effect.

Similar Phenomena and Possible Mechanisms

We are not aware of any other reports in the literature of transgenes inhibiting homologous gene expression, either in plants or in other higher organisms. There are, however, a number of examples of similar allelic interactions that could be related mechanistically to co-suppression. One example is at the CHS-encoding *nivea* locus in *Antirrhinum majus* in which a semi-dominant allele is able to inhibit the expression of the wild-type allele *in trans* (Coen and Carpenter, 1988). This effect at the *Antirrhinum* CHS locus, taken together with the petunia CHS co-suppression effect, suggests that it would be worthwhile to investigate the basis of another dominant CHS allele, the *C2-Idf* allele in maize (Coe and Neuffer, 1977).

A well-known allelic interaction in plants that may be relevant to understanding co-suppression is the phenomenon of paramutation. Paramutation is defined as "an interaction between alleles that leads to directed, heritable

change at a locus with high frequency . . . within the time span of a generation" (Brink, 1973). Paramutation may occur even when alleles are on separate chromosomes in non-heterochromatic regions (Brink, 1964). An apparent distinction between paramutation and co-suppression is that co-suppression does not persist after the transgene segregates away, although we have tested this in only one population so far. Nevertheless, if the transgene were the equivalent of a paramutable allele and the endogenous gene were the equivalent of a paramutagenic allele, co-suppression could be the same as paramutation. To test this, a CHS null mutant would be required; the persistence of the co-suppression effect on the transgene in a plant homozygous for the recessive CHS null allele then could be determined.

The erratic and reversible nature of the CHS transgene effect suggests the involvement of methylation. For instance, the cycling of maize transposable elements between active and inactive states is determined by the methylation state of the element (Chandler and Walbot, 1986; Chomet et al., 1987; Federoff, 1989). Also, an epistatic interaction between T-DNAs reported by Matzke et al. (1989) is correlated with methylation of the genes involved. This interaction is between nonhomologous transgenes located on different T-DNA elements that do, however, share homology near the transgenes. By contrast with the work reported here, only the genes carried by one of the T-DNA elements were found to be inhibited.

An important example of interactions between homologous sequences over large distances is the repeat induced point mutation (RIP) phenomenon in *Neurospora* (Selker et al., 1987). RIP is characterized by instability of duplicated sequences during the sexual phase. Both endogenous (original copy) and introduced (second copy) DNA sequences become rearranged and methylated and accumulate mutations at an extremely high frequency. If, before the introduction of a DNA sequence, the homologous DNA sequence is deleted, the unique introduced DNA is unaltered by RIP. Thus, the RIP system is capable of recognizing that a sequence has become duplicated and acts to modify it. The observation that *both* copies of a duplicated sequence are subject to RIP parallels the observation of co-suppression of both introduced and endogenous copies of CHS and DFR. There is no evidence for induced point mutations associated with co-suppression, and, in co-suppression, the interaction between homologous sequences must occur somatically. However, we would suggest that the possible relevance of RIP to understanding co-suppression is the ability of the RIP system to recognize DNA sequences located at distant positions in the genome. Thus, RIP provides a precedent for the idea that homologous DNA sequences can interact in some fashion, even when located at a distance from each other.

Another kind of allelic interaction that has been intensely investigated in recent years is the "transvection effect" at the *White* and other loci in *Drosophila*, whereby one allele can influence the expression of a homologous allele, even if the two alleles are on different chromosomes (Wu and Goldberg, 1989). Wu and Goldberg review a number of models for transvection; perhaps the most interesting hypothesis is that *trans* effects might be mediated by cooperative binding by a regulatory protein (the product of the *zeste* gene) to multiple binding sites in the genome. There is no evidence for such a mechanism operating in co-suppression.

METHODS

Plasmid Constructions

DNA manipulations were carried out as described in Maniatis et al. (1982). The plasmid pcPE1, which contains a nearly full-length CHS cDNA clone from *Petunia hybrida* L. (petunia), was kindly provided by H.J. Reif, Max-Planck-Institut, Köln. The EcoRI fragment containing the complete coding sequence for CHS was recloned into the EcoRI site of p*UC119* for site-directed mutagenesis using the method of Kunkel (1985). Two synthetic oligonucleotides, synthesized on an Applied Biosystems 381A DNA synthesizer, were used to introduce two new restriction sites, HpaI and NcoI, at the beginning and a BamHI site at the end of the coding sequence. In vitro synthesized, double-stranded DNA was transformed into *Escherichia coli* strain DH-1, and the plasmid that contained all three introduced restriction endonuclease sites was identified and designated as pFLG5571. Plasmid pFLG5571 DNA was cleaved to completion with BamHI and then cleaved with NcoI under conditions to give a partial, incomplete digestion of the DNA because a second NcoI site lies within the CHS coding sequence. The 1170-bp NcoI-BamHI fragment containing the CHS coding sequence was ligated at the NcoI site to a modified cauliflower mosaic virus 35S promoter (Harpster et al., 1988) and at the BamHI site to a polyadenylation signal sequence from the nopaline synthase (*nos*) gene (Depicker et al., 1982). This plasmid was designated pFLG5634. The partial NcoI-BamHI fragment was ligated to the promoter and polyadenylation signal sequence in the antisense orientation to give plasmid pFLG5646. The entire 35S promoter-CHS-*nos* polyadenylation sequence fusion in plasmids pFLG5634 (sense) and pFLG5646 (antisense) was contained within a BglII-HindIII fragment that was ligated into the BamHI and HindIII sites in the binary vector pJJ3942. Plasmid pJJ3942 is based on the broad host range cloning vector pRK290 (Ditta et al., 1980) and contains a neomycin phosphotransferase II coding sequence fused at the 5′ end to a nopaline synthase promoter and at the 3′ end to an octopine synthase polyadenylation signal sequence between the left and right T-DNA borders. pJJ3942 contains an enhancer-like sequence from the cauliflower mosaic virus 35S promoter next to the T-DNA right border. The resulting plasmids were designated as pFLG5972 (sense) and pFLG7010 (antisense) and were transferred to the disarmed *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al., 1983) using a triparental mating (Ditta et al., 1980).

Plasmid pFLG3240 was constructed for use in RNase protection analyses. Plasmid pFLG5634 was digested to completion with XhoI and BclI. The 156-bp XhoI-BclI fragment containing the Cab22L untranslated leader sequence and 97 bases of the CHS

coding sequence was ligated to the XhoI and BamHI sites in the polylinker of pBluescript KS+ (Stratagene). The plasmid containing this fragment was designated as pFLG3240.

Plant Transformation

Petunia hybrida varieties V26, R18, M1, and R27 were obtained from Anton Gerats, Department of Genetics, Free University, Amsterdam. *P. hybrida* "Pink Cascade" was obtained from Michael Reid, Department of Environmental Horticulture, University of California, Davis. Petunia plants were grown from surface-sterilized seed on sterile, solidified agar medium of one-tenth the concentration of medium MS (Murashige and Skoog, 1962) supplemented with 0.5% sucrose. After germination, seedling tops were excised by cutting in the hypocotyl region and transferred to MS with 3% sucrose. Plants were maintained at 28°C under cool-white fluorescent light at 4000 lux to 5000 lux, 16 hr/day. About 6 weeks after planting (day 0), leaves were excised, cut with a scalpel blade into pieces about 5 mm square, and inoculated with *A. tumefaciens* (pFLG5972 or pFLG7010) that had been grown overnight in MinA medium supplemented to 0.2% glucose and adjusted to 0.1 A_{550} unit to 0.2 A_{550} unit. Inoculated leaf pieces were placed on incubation medium [basal MS medium (MS with 3% sucrose and B5 vitamins) plus 75 μM to 100 μM acetosyringone, 1 mg of benzyladenine/L, and 0.2 mg of indoleacetic acid/L] for 2 days in a sterile transfer hood at room temperature (approximately 22°C). On day 2, 25 mL to 30 mL of liquid basal MS medium plus cefotaxime (500 mg/L) was added to the plates. Plates were then swirled at 70 rpm to 100 rpm for 30 min to 60 min. Leaf pieces were transferred with the upper epidermis facing up on selection medium (basal MS with benzyladenine at 1 mg/L), indoleacetic acid at 0.2 mg/L, vancomycin at 100 mg/L, kanamycin at 300 mg/L). The plates were sealed with parafilm and incubated at 24°C under moderate light (3000 lux to 5000 lux). On day 14, leaf pieces were transferred to fresh selection medium. On day 28, calli were excised from leaf pieces and transferred to fresh selection medium, and shoots were excised and transferred to hormone-less medium (basal MS plus vancomycin at 100 mg/L and kanamycin at 100 mg/L). On day 42 and following, shoots were excised from calli and transferred to hormone-less medium. After shoot elongation, shoots were excised and dipped in naphthalene acetic acid at 0.1 mg/L for root development. After rooting, plantlets were transplanted to soil and grown in a greenhouse. Constructs pFLG5972 and pFLG7010 were introduced to Pink Cascade, R18, and V26.

Preparation and Analysis of RNA

Flowers were harvested from plants and frozen in liquid nitrogen. Frozen tissue (100 mg to 400 mg) was ground to a fine powder in a mortar with liquid nitrogen. The powder was transferred to a 1.5-mL Eppendorf microcentrifuge tube containing 0.150 mL of phenol saturated with Tris, pH 7.5, 0.5 mL of 100 mM NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA, 1% SDS, and vortexed. Chloroform:isoamyl alcohol (0.25 mL, 24:1) was added and the solution vortexed. The aqueous and organic layers were separated by centrifugation, the aqueous phase was transferred to a clean Eppendorf tube, and the RNA was precipitated by the addition of an equal volumn of 4 M lithium acetate. After 3 hr on ice, the solution was centrifuged, the RNA pellet was resuspended in water, and the RNA was precipitated in the presence of 0.3 M sodium acetate and 2.5 volumes of ethanol. The RNA pellet was resuspended in water.

RNase Protections

Steady-state messenger RNA levels were analyzed by the RNase protection method. A 221-nucleotide radiolabeled antisense CabL-CHS RNA was transcribed in vitro by T7 polymerase from plasmid pFLG3240 following the RNA transcription protocol provided by Stratagene. The synthesized RNA probe was purified by precipitating the RNA with 100% ethanol in the presence of 2 M ammonium acetate, followed by precipitation with 100% ethanol in the presence of 0.3 M sodium acetate. The RNA pellet was dissolved in 25 μL of water. Five micrograms of petal RNA was annealed with 1×10^6 cpm labeled RNA probe as described in a protocol provided by Promega Biotec [protocol 3, RNase ("S1 type") mapping]. Hybridization was done overnight at 42°C. RNase A and RNase T1 digestion was performed as described in the Promega Biotec protocol. A similar RNase protection procedure is described by Gilman (1989). The samples were analyzed on a denaturing 6% acrylamide, 8 M urea gel. After incubation with RNase A and RNase T1, three different protected fragments remained: a 94-nucleotide fragment representing the endogenous CHS mRNA, a 157-nucleotide fragment representing the introduced CHS transcript, and a 60-nucleotide fragment representing the CabL untranslated 5′ sequence.

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